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Screening of Extract Secondary Metabolites of Bacteria Which Have Symbiosis with Sponges from Central Tapanuli Bakar Island as an Antibacteria

Mirnawati*, Rasyidah, Ulfayani Mayasari, Endang Sulystiarini Gultom
Biology Study Program, Faculty of Science and Technology, North Sumatra State Islamic University, Jl. Lapangan Golf No. 120, Medan
*Corresponding Author: mirnawati463@gmail.com

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ABSTRACT

This study aims to determine the presence of antibacterial-producing bacteria from sponges from Bakar Island, Central Tapanuli Regency and to determine the antibacterial activity of methanol extracts from potential bacterial isolates against *Staphylococcus aureus* and *Escherichiacoli*. 21 pure isolates were found and it was known that 9 isolates produced antibacterial against *S. aureus* and 4 isolates against *E. coli*. Bacterial isolates with the highest inhibition zone against both *S.aureus* and *E. coli* were found in isolates with code SP20 with each large inhibition zone are 14.5 mm and 16.04 mm. Then microscopic observations were carried out, namely gram staining and were obtained 8 isolates were gram positive, and 1 gram was negative. The biochemical test showed that SP20 showed positive results for the Motility, TSIA and Catalase Test but negative for Citrate Test. Bacterial isolates withthe code SP20 had the largest inhibition zone so that these isolates were to be extracted.The resultsof the screening test showed that the secondary metabolitesof the sponge symbiont bacterial extract from Bakar Island were positive for flavonoids and saponins. And then test the activity of secondary metabolites with 3 concentrations, 10%, 20%, and 30%.The results showed that the best concentration of the test bacteria was *S. aureus* at a concentration of 30% with an inhibition zone of 3.08 mm and *E. coli* at 5.04 mm.
Keyword: *Sponge, Bacteria, Antibacterial, Secondary Metabolites*

ABSTRAK

Penelitian ini bertujuan untuk mengetahui adanya bakteri penghasil antibakteri dari spons asal Pulau Bakar Kabupaten Tapanuli Tengah serta untuk mengetahui aktivitas antibakteri ekstrak metanol dari isolat bakteri potensial terhadap bakteri uji *Staphylococcus aureus* dan *Escherichia coli*. 21 isolat murni ditemukan dan diketahui 9 isolat merupakan bakteri penghasil antibakteri terhadap *S. aureus* dan 4 isolat terhadap *E. coli*. Isolat bakteri dengan zona hambat paling tinggi terhadap kedua bakteri uji *S. aureus* dan *E. coli* adalah SP20 dengan besar zona hambat masing-masing 14,5 mm dan 16,04 mm. Selanjutnya dilakukan pengamatan mikroskopik yaitu pewarnaan gram dan diperoleh 8 isolat bakteri gram positif, dan 1 isolat bakteri gram negatif. Uji Biokimia isolat SP20 menunjukkkan hasil positif pada Uji Motilitas, Uji TSIA, dan Uji Katalase namun hasil negatif terhadap Uji Sitrat. Isolat bakteri dengan kode SP20 memiliki zona hambat paling besar sehingga isolat tersebut yang akan di ekstraksi, dari hasil uji skrining menunjukkan bahwa metabolit sekunder ekstrak bakteri simbion spons asal Pulau Bakar positif terhadap flavonoid dan saponin. Dan selanjutnya uji aktivitas metabolit sekunder dengan 3 konsentrasi yaitu 10%, 20%, dan 30%. Hasil penelitian menunjukkan bahwa konsentrasi paling baik terhadap bakteri uji *S.aureus* pada konsentrasi 30% dengan zona hambat 3,08 mm dan *E. coli* sebesar 5,04 mm.
Keyword: Spons, Bakteri, Antibakteri, Metabolit Sekuder

1. Introduction

Central Tapauli Regency is one of the districts on the West Coast of North Sumatra. It is located on the west coast of Sumatra Island with a coastline of 200 km and an altitude ranging from 0–1,266 meters above sea level (BPS, 2019). Bakar Island is one of the islands in Badiri sub-district, Central Tapanuli Regency, on this island there is marine biota, one of which is sponges. This sponge has not been utilized by the local community because there is still minimal information about marine biota, especially sponges.

Sponges are multicellular animals with 15,000 known species and only 1% inhabit fresh water areas [1]. Sponges are one of the biota that make up coral reefs which have bioactive potential [12]. Sponges live in symbiosis with various types of bacteria. Bacteria in symbiosis with sponges will most likely produce the same bioactive substances as their hosts. These marine animals contain active compounds whose active percentage is greater than the compounds produced by land plants [8].

According to [5]. sponges can produce alkaloid, terpenoid and steroid bioactive compounds which have antibacterial properties. Antibacterials are needed to treat infections caused by bacteria. [3]. succeeded in isolating bacteria that have the potential to act as antibacterials that live in association with the sponge *Haliclona* sp. and *Axinellid* sp. In the research results of [8]. they succeeded in isolating bacteria that have the potential to act as antibacterials that live in association with the *Petrosia* sp sponge which is able to inhibit the growth of *Escherichia coli* and *Staphylooccus aureus* bacteria. Therefore, this research aims to determine the presence of antibacterial producing bacteria in sponges from Bakar Island, Central Tapanuli.

2. Research methods

2.1 *Sampling*

Fresh samples are taken directly from the sea (5-10 meters) above sea level using scuba diving equipment, then washed and cleaned then put in plastic containing oxygen, the samples are then stored in an ice box until used. Before the sample is used, the sample is dried and crushed using a *mortar stamper*.

2.2 *Isolation of Symbiotic Bacteria*

One gram of sponge sample was placed in a test tube containing 9 ml of sterile seawater. Dilution of the bacterial suspension was carried out in stages up to a dilution of 10−8 [9]. After that, 1 ml of each dilution result was taken to be inoculated on MA media aseptically. Then incubated at 37°C in an incubator for 1 x 24 hours [11]. The isolate that has been obtained is then purified.

2.3 *Antibacterial Activity Screening*

After obtaining the pure isolate, the antibacterial activity test method was carried out against *Staphylococcus aureus* and *Eschericia coli* as test bacteria. 200 µl of the test bacterial suspension was taken and put into 15 ml of MHA which had begun to cool, then poured into a petri dish and left to freeze. After that, a paper disc that has been soaked with a suspension of sponge bacteria to be tested is placed onto the media, then incubated for 24 hours [6]. The size of the inhibition zone that appears is measured and classified into the Davis Stout category, namely ≥ 20 mm = very strong, 10–20 mm = strong, 5–10 mm = medium and ≤ 5 mm = weak.

2.4 *Bacterial Identification*

Identification of bacteria that have the potential to produce antibacterials is carried out using physiological tests and morphological observations including color, shape, edges and elevation of the colony. Physiological testing of bacteria includes staining gram, catalase test, citrate test, motility test and TSIA test.

2.5 *Extraction*

Maceration is carried out by culturing bacterial isolates into NB media for 3 days, then adding methanol PA solvent in a ratio of 1:1 for 3 days, then filtering with filter paper, the resulting macerated extract is collected and evaporated to separate the solvent [9]. Evaporation is carried out using a Rotary Evaporator analysis tool at a temperature of 45-50°C, until the solvent has evaporated, so that a thick extract of bacteria is obtained [6].

2.6 *Secondary Metabolite Activity Test*

200 µl of the test bacterial suspension was taken and put into 15 ml of MHA which had begun to cool, then poured into a petri dish and left to freeze. After that, a disc of paper is placed in the designated area and then dripped with sample extract at a concentration of 10%, 20% and 30%, then incubated for 24

hours. To determine the effectiveness of the test bacteria, a comparison antibiotic was used, namely amoxilin with a concentration of 1389 ppm [14].

2.7 *Data analysis*

Data from testing results of antibacterial sponge symbiont bacterial isolates against *Staphylococcus aureus* and *Escherichia coli* were analyzed descriptively with results tables and supporting images.

3. Results and Discussion

The sponge sample obtained from the waters of Bakar Island, Central Tapanuli Regency has a purplish blue color and a rough texture which can be seen in Figure 1. The pH of the sea water at the sampling location is 6, the salinity is 36 ppt and the temperature is 27^{o}C. The position of the sample collection location based on GPS data is N.1.35.17.N.98.41.56.E. Measurement of the environmental conditions of the sea water at the location Sampling aims to create suitable environmental conditions for testing symbiont bacteria so that the symbiont bacteria can survive.

Figure 1 . Sponge from Bakar Island, Central Tapanuli Regency

Morphological Characteristics of Bacterial Isolates

Based on the results of isolation using the pour plate method and inoculation using the continuous scratch method, characteristics were found in the form of size, shape, elevation, margin and color of the colonies of the 21 isolates, namely round and irregular shapes. The edges of the colony are entire, undulate, and lobate, have flat, raised, and convex elevations, and have varying sizes, some are large, moserate, small, and punctiform. And all isolates have a white color. The colony morphology of bacterial isolates found in this study is in accordance with the statement [2]. that bacteria are generally circular, irregular, filamentous, rhizoid in shape. Elevations are raized, convex, flat, umbonate. The edges are entire, undulate, and lobate.

Antibacterial Activity against Staphylococcus aureus and Escherichia coli

Sponge isolates from Bakar Island, Central Tapanuli Regency are known to have antibacterial activity against the two test bacteria used. This is proven by the existence of an inhibition zone that appears around the disc paper. The size of the clear zone produced by each bacteria can be seen in table 1. Based on the results of the antibacterial test, the ability of bacterial isolates associated with the sponge from Bakar Island is that there are 9 isolates, namely SP1, SP3, SP4, SP5, SP11, SP13, SP14, SP18 and SP20 which have antibacterial potential in inhibiting the growth of the test bacteria S. aureus and 4 isolates namely SP4, SP13, SP18, and SP20 which are able to inhibit the growth of *E. coli* bacteria.

Table 1. Results of antibacterial tests against Staphylococcus aureus and Escherichia coli

Isoate	***Staphylococcus aureus***		***Escherichia coli***	
	Diameter	**Criteria**	**Diameter**	**Criteria**
SP1	0.5mm	Weak	-	-
SP2	-	-	-	-
SP3	1.5mm	Weak	-	-

SP4	7.05mm	Medium	11.5	Strong
SP5	1.0mm	Weak	-	-
SP6	-	-	-	-
SP7	-	-	-	-
SP8	-	-	-	-
SP9	-	-	-	-
SP10	-	-	-	-
SP11	1.0mm	Weak	-	-
SP12	-	-	-	-
SP13	8.06	Medium	13.04	Strong
SP14	0.05	Weak	-	-
SP15	-	-	-	-
SP16	-	-	-	-
SP17	-	-	-	-
SP18	7.5mm	Medium	9.05	Medium
SP19	-	-	-	-
SP20	14.5mm	Strong	16.04	Strong
SP21	-	-	-	-

The diameter of the clear zone produced by a potential antimicrobial isolate indicates that the bacteria produce a chemical composition that inhibits colonization by other microbes. This chemical composition has antimicrobial activity which can be in the form of antibiotics, pigments, toxins and enzyme inhibitors. The mechanism of inhibition of bacterial growth by antimicrobial compounds can occur in the form of cell damage by inhibiting the formation of cell walls, damaging the permeability of the cytoplasmic membrane, causing the release of fluid in the cell, changing protein molecules and nucleic acids and proteins [5].

The greatest antibacterial potential was shown by isolate SP20 which had an inhibition zone against bacteria S. aureus of 14.5 mm and against E. coli bacteria of 16.04 mm based on the inhibition zone, isolate SP20 was chosen as the isolate with the most potential against pathogenic bacteria. Next, the SP20 isolate was cultivated in NB (Nutrien Broth) media on a large scale to obtain secondary metabolites. The difference in the diameter of the inhibition zone for each bacteria is influenced by the level of antimicrobial fermentation of each isolate in producing secondary metabolites. This inhibitory ability is related to bacterial colonization in the form of the ability to attach, motility and chemotaxis of bacteria to nutrients and organic material [14].

Bacterial Identification

The isolate with the highest inhibition zone was then identified to determine its genus. Identification is carried out microscopically and physiologically. Microscopic observations were carried out through gram staining. Based on gram determination, isolates SP1, SP3, SP4, SP5, SP11, SP13, SP14, and SP20 are classified as Gram positive bacteria, all of which are in the form of bacilli (rods) except for SP5 which is in the form of a coccus. This is due to the bacteria's ability to bind the main dye (crystal violet) which is so strong that it is more dominant than the opposing dye (safranin). Meanwhile, the SP18 bacterial isolate is classified as a Gram-negative bacteria and is in the form of bacilli (rods). In general, marine bacteria found are rod-shaped because they have flagella (75-85%) to move in the water. Meanwhile, coccus bacteria have the advantage of bonding with each other, forming a strong (solid) surface due to the presence of slimy material so that the cells are linked together. This method allows bacteria to form a surface layer which results in the bacteria being able to live in symbiosis [10].

Observation of physiological tests, including citrate test, catalase test, motility test and TSIA test. The citrate test aims to find out whether the bacteria use citrate as a carbon source. Positive results if there is a change in media color from green to blue [9]. The SP20 isolate citrate test showed negative results. The catalase test is useful for identifying bacteria that can produce the catalase enzyme. Positive results were

indicated by the presence of bubbles after the isolate was dripped with 3% H2O2 solution. SP20 isolate showed positive results.

Apart from that, a motility test is also carried out to determine the ability of bacteria to move to get their food. SP20 showed positive results as indicated by the presence of spreads around the area of the oss puncture. The Triple Sugar Iron Agar (TSIA) test is a biochemical test which aims to find out whether the bacterial isolates found are included a group of bacteria that can ferment glucose to form acid or not. The SP20 bacterial isolate belongs to the group of bacteria that are wet/acid (K/A), where the top part remains red and the bottom of the media turns yellow.

Research carried out on bacteria with code SP20 is included in the genus Bacillus, bacillus bacteria have the characteristics of a round and small round shape with flat and rocky edges, are white in color, the bacterial cells are gram positive, are facultatively anaerobic, are able to ferment sucrose, and some produce gases in their metabolism, some use citrate as a carbon source and are motile. Classification of these bacteria Manual of Determinative Bacteriology $8th$ based on Bergey's Edition of the bacterial identification book.

Symbiotic Bacterial Metabolite Production

Methanol was used as a solvent in the extraction process in this research because methanol can dissolve polar and non-polar compounds. Methanol solvent can dissolve alkaloid compounds, tannins, saponins, steroids and flavonoids [12]. According to [13]. methanol solvent is a universal solvent that easily enters cells through the cell walls of the material so that methanol can attract most of the chemical compounds contained in the cells to dissolve in the solvent without destroying the compound content.

The results of the screening test showed that the secondary metabolites of sponge symbiont bacteria from Bakar Island were positive for flavonoids and saponins but negative for tannins, steroids and alkaloids, this was indicated by the absence of color changes after being given Lieberman-Burchard and Mayer reagents. [6]. reported that each sea sponge does not always have the same secondary metabolites as other sponges, environmental conditions greatly influence the formation of secondary metabolite content.

Table 2. Secondary Metabolite Compound Test Results

Compound	Results	description
Alkaloid	—	No white or yellow ish precipitate is formed after adding Meyer's reagent.
Flavonoids	+	A yellow color forms on the top layer, indicating positive flavonoids.
Saponins	+	Stable foam is formed after adding concentrated HCL.
Tannin	—	No red or blackish deposits.
Steroid	—	There is no color change on the top layer.

Positive saponin compounds were identified in the methanol extract of sponge symbiont bacteria. Saponin has the ability to act as an antibacterial because it can damage the surface of bacterial cell walls and cause leakage of proteins and enzymes from within microbial cells [5]. Apart from that, the presence of flavonoid compounds was also identified as indicated by the formation of a red color. [11]. the ability of flavonoid compounds to affect bacteria is by interfering with the synthesis of bacterial cell membranes through inhibition which results in the merging of glycan chains so that the cell membrane and peptidoglycan weaken and then cause damage which can result in lysis of the cell wall.

Antibacterial Activity of Sponge Secondary Metabolites

The sponge symbiont bacterial metabolite extract obtained was made into 3 different concentrations, namely 10%, 20% and 30%. The inhibitory ability of the sponge symbiont bacterial extract from Bakar Island against the two test bacteria S. aureus and E. coli, shows that the greater the concentration given, the

greater the inhibitory area formed. This is thought to be due to the addition of greater active compounds with the addition of higher concentrations. This is comparable with research [4]. that the active compounds contained in the extract with each increase in concentration will be greater so that the work force in inhibiting bacterial growth is more effective.

Table 3. Antibacterial Activity of Sponge Secondary Metabolites

	S.aureus		***E.coli***	
	U1	**U2**	**U1**	**U2**
Control +	28.01	26.07	18.02	24.01
Control–	—	—	—	—
10%	—	—	—	—
20%	1.07	—	—	—
30%	3.07	3.5	6.04	5.5

An antibacterial compound is said to have high activity against microbes if the minimum inhibitory concentration value is low but produces a large inhibitory power. The observation results showed that the antibacterial activity was greatest at a concentration of 30% against the test bacteria *Staphylococcus aureus* and *Escherichia coli*. The positive control used amoxicillin, which had an effect on both test bacteria and its inhibitory activity was in the strong category. On the other hand, the negative control did not show any inhibitory power, thus indicating that the control used had no effect on the antibacterial test.

According to David Stout, the antibiotic strength of SP20 isolate extract at a concentration of 30% is included in the weak category for inhibiting the growth of pathogenic bacteria *Staphylococcus aureus* and *Escherichia coli*. Factors that influence the size of the inhibition area are organism sensitivity, culture medium, incubation conditions and agar diffusion speed. Factors that influence agar diffusion speed, concentration of microorganisms, media composition, incubation temperature and incubation time [16]. A concentration of 30% with a weak inhibitory power category is an indication of a capable antibacterial concentration inhibits the growth of microorganisms and also provides instructions regarding the dosage required in the treatment of diseases caused by *Staphylococcus aureus* and *Escherichia coli* bacteria.

4. Conclusion

Based on the research results, it can be concluded that:

1. Sponges from Bakar Island, Central Tapanuli contain antibacterial producing bacteria.
2. Isolates SP4, SP13, SP18, and SP20 have antibacterial activity against both test bacteria *S. aureus* and *E. coli*
3. A genus of bacteria originating from sponges from Bakar Island, Central Tapanuli which have the potential to produce antibacterials, namely the genus Bacillus
4. SP20 extract contains secondary metabolite compounds, namely saponins and flavonoids
5. Secondary metabolite extracts from sponge symbiont bacteria have the potential to inhibit the growth of pathogenic bacteria *S. aureus* and *E. coli*.

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